RESEARCH PAPER

Regulation of cell cycle activity in the embryo of barley seeds during germination as related to grain hydration

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Abstract

Various studies indicate that cell division is a post-germination phenomenon, with radicle protrusion occurring by cell elongation, while others demonstrate that induction of the cell cycle occurs in osmo-conditioned seeds prior to radicle growth. The aim of the present work was to investigate the occurrence of the cell cycle during germination as related to grain hydration, using: (i) a flow cytometry technique to estimate the percentage of cell nuclei in G1 and G2 phases of the cell cycle; and (ii) reverse transcription-PCR (RT-PCR) in order to characterize the expression of the genes encoding cyclin-dependent kinases (CDKA1, CDKB1, and CDKD1) and cyclins (CYCA3, CYCB1, and CYCD4), the main genes involved in the cell cycle and its regulation. Radicle tips of embryos were isolated from seeds placed for various times on water at 30°C and from grains partially hydrated at moisture contents ranging from 11% to 51% fresh weight (FW), which prevent radicle elongation. Abscisic acid (ABA) contents of the embryos during seed germination at 30°C and after 48 h of partial hydration were also measured. In dry embryos, cells are mostly arrested in the G1 phase of the cell cycle (82%), the remaining cells being in the G2 phase, and the ABA content of the embryo was 432.7 ng g⁻¹ dry weight (DW). Seed imbibition was associated with a sharp decrease in ABA content as early as 5 h, while the cell cycle reactivation was a late process taking place 4–6 h prior to radicle protrusion. Hydration of seeds resulted in a decrease in embryo ABA content, but it remained at a high level (207–273 ng g⁻¹ DW) even after 48 h at 0.41–0.51 g H₂O g⁻¹ FW. The cell population of the radicle tips in the G2 phase of the cell cycle, i.e. 4C nuclei, increased from 9% up to 34% at a moisture content of 51% FW. In dry seeds, CDKA1 and CDKD1 mRNAs were present at low levels, but transcripts of CDKB1, CYCA3, CYCB1, and CYCD4 were not detected. Radicle protrusion was associated with a higher expression of CDKA1, CDKB1, CYCA3, and CYCB1. Blockage of germination of partially hydrated grains resulted in a reduction in the expression of CDKA1 and CDKB1, and of CYCA3 and CYCB1, and in a reinforcement of that of CDKD1 and CYCD4. Patterns of gene expression show differential sensitivity of the genes studied to hydration of the grain. They will be discussed with regard to embryo ABA content and embryo sensitivity to ABA.

Key words: Abscisic acid, barley, cell cycle, cyclin, cyclin-dependent kinase, germination, Hordeum vulgare L., water content.

Introduction

Seed germination is a physiological process by which the embryo resumes growth following three phases: imbibition (phase I), germination sensu stricto (the true germination process, phase II), and growth (phase III) (Côme, 1980; Bewley, 1997). Germination is considered to be completed when the radicle protrudes from the covering structures. Water uptake results in the resumption of respiratory activity and protein synthesis using
extant mRNAs, and phase II, the most important phase, is associated with various cellular and biochemical events including DNA repair, protein synthesis relying on the translation of new RNA, changes in soluble sugars, etc. (Bray, 1995; Bewley, 1997).

The involvement of cell division activation in germination remains debatable, since various data indicate that radicle protrusion does not require mitotic activity (Baiza et al., 1989), whereas others demonstrate that cell division has been found prior to radicle protrusion in tobacco, tomato, and Arabidopsis (De Castro et al., 2000; Barróco et al., 2005; Masubelele et al., 2005). Studies on the effects of priming on DNA replication have also given inconsistent results. Only low DNA synthesis is found during priming in leek (Bray et al., 1989) and tomato (Coolbear and Grierson, 1979) seeds. In contrast, studies using flow cytometry have demonstrated that DNA replication is initiated in the radicle tip cells during hydro- or osmopriming of seeds of tomato (Bino et al., 1992; Lanteri et al., 1994; Özbingöl et al., 1999), pepper (Lanteri et al., 1994; Saracco et al., 1995), and sugarbeet (Redfearn and Osborne, 1997; Sliwinska, 2000).

Absisic acid (ABA) plays a major role in regulating seed germination and dormancy (Bewley, 1997). It has been shown to inhibit DNA replication in cultured embryo axis of pea (Levi et al., 1993) and during early germination of Avena fatua seeds (Elder and Osborne, 1993). In contrast, Liu et al. (1994) demonstrate that ABA inhibits seed germination in the wild-type and the ABA-deficient mutant siti of tomato, while nuclear replication was not affected. In non-dormant seeds, which are less sensitive to ABA than dormant seeds (Corbineau and Côme, 2000, 2003; Benech-Arnold et al., 2006), the water content of the embryo or water potential might play a primordial role in controlling cell cycle progression and radicle protrusion (Bradford, 1995). Radicle elongation is generally inhibited at –1.0 to –1.5 MPa depending on the species. In addition, induction of DNA replication during controlled imbibition (i.e. during priming) is inversely related to the water content of the seeds (Lanteri et al., 1994; Özbingöl et al., 1999). In tomato, for example, the percentage of 4C nuclei remains close to the initial value measured in dry unprimed seeds when osmopriming is performed at –2.0 MPa, i.e. at a seed moisture content of <68% on a dry weight (DW) basis (~40% on a fresh weight (FW) basis).

Cell cycle progression is controlled by reversible phosphorylation by protein kinases and phosphatases, and in particular by the activity of cyclin-dependent kinase (CDK) (Dewitte and Murray, 2003). CDK activity is further modulated by various regulatory proteins such as cyclins (Morgan, 1997). Many CDK and cyclin homologues have been found in plants. CDKs are classified into five groups from CDKA to CDKE (Joubès et al., 2000). CDKs from the CDKA group exhibit a PSTAIRE motif in their amino acid sequence involved in their interaction with cyclin proteins. They are considered as orthologues of Cdc2, a single CDK in fission yeast. Plants also have an additional CDK group, CDKB, containing another specific protein motif PPTA/TLRE and having the unusual property of their expression being cell cycle regulated (Hirayama et al., 1991; Fobert et al., 1996; Magyar et al., 1997). Plants, as could be expected, contain regulatory proteins associated with kinases, cyclins, and others (Renaudin et al., 1996). Their classification is similar to that of mammalian cyclins, most plant cyclins being related to A-, B-, and D-type cyclins (De Veylder et al., 1999). Most cyclins contain a destruction box which gives them a short life span and an important regulatory role in cell cycle control. A-type cyclins are expressed in the S, G2, and M phases depending on their subtype, while B-type cyclins are strictly expressed in the late G2 and M phases (Fuerst et al., 1996; Mironov et al., 1999). D-type cyclins have a different pattern of expression, being mainly involved in the G1 to S transition (Renaudin et al., 1996) or in the G2 to M transition (Menges et al., 2005).

The objectives of this study were (i) to investigate whether the cell cycle progression is associated with germination of non-dormant barley seeds as related to water content; (ii) to determine the effects of water content on the expression of the main genes involved in the cell cycle activity; and (iii) to study whether (ABA) interferes with the progression of the cell cycle.

Materials and methods

Plant material

Barley (Hordeum vulgare L., cv. Pewter) seeds were harvested in July 2004 at the end of the maturation drying phase, i.e. when their water content reached 0.10–0.12 g H2O g–1 FW, and were provided by the ‘Coope´rative de Toury’ (Eure et Loir, France). Experiments were carried out with grains stored dry in the open air for 3 months at 25 °C in order to break their dormancy (Corbineau and Côme, 1996). After such an after-ripening treatment, seeds were non-dormant and able to germinate at 30 °C, the temperature at which dormancy is expressed (Corbineau and Côme, 1996).

Germination assays

Germination assays were performed at 30 °C in darkness, in three replicates of 50 grains placed in 9 cm diameter Petri dishes on a layer of cotton wool imbibed with deionized water. A grain was regarded as germinated when the radicle had protruded through the seed-covering structures. Germination counts were conducted regularly for 7 d. Results presented correspond to the means of the germination percentages obtained in three replicates ±SD.

Experiments carried out with 50 mM hydroxyurea, an inhibitor of the S phase (Planchais et al., 2000), were done by incubating seeds in the presence of this compound from the beginning of imbibition.
Seed treatment and water content determination

Seeds (~10 g) were placed in tightly closed 125 ml flasks with different amounts of water to obtain various embryo water contents ranging from 0.11 to 0.65 g H₂O g⁻¹ FW. Water equilibrium was carried out by rotating the flasks at 30 °C on a roller at 60 rotations min⁻¹ for 24 h. Thirty isolated embryos were weighed and then oven-dried at 105 °C for 48 h for determination of the DW and calculation of the water content. Water content was calculated on a FW basis. Results are expressed as g H₂O g⁻¹ FW and correspond to the means of the values obtained in three replicates of 30 embryos.

Flow cytometry

Amounts of nuclear DNA were quantified using radicles of embryos isolated from grains incubated at 30 °C for various times on water or at different water contents. Samples of 15 radicles were chopped on ice with a razor blade in 400 μl of nuclear isolation buffer [20 mM MOPS, 30 mM Na citrate, 45 mM MgCl₂, 1% Triton X-100 (w/v)] adapted from Galbraith et al. (1983). The suspension was sieved through a 48 μm nylon mesh. After 15 min of digestion with RNase (1 μg 10⁻¹ μl⁻¹) at room temperature, the samples were stained with propidium iodide (1 mg ml⁻¹) to allow measurement of the amount of nuclear DNA by fluorescence. DNA analyses were performed using an EPICS XL-MCL flow cytometer (Beckman-Coulter, Miami, FL, USA) equipped with an argon ion laser at 488 nm, and fluorescence was detected over a range 605–635 nm.

The amount of DNA is proportional to the fluorescent signal and is expressed as an arbitrary C value, in which the 1C value represents the amount of DNA of the unreplicated haploid chromosome complement. Three experiments were performed independently, and the results presented in Fig. 1B correspond to a representative single experiment. The frequency of 2C and 4C nuclei was calculated as [2C nuclei/(2C nuclei+4C nuclei)]×100 and [4C nuclei/(2C nuclei+4C nuclei)]×100, respectively. The average variation between two measurements of a population of nuclei is ~3%. The populations of 2C and 4C nuclei were measured on 10 000 nuclei.

RNA extraction

Embryos were isolated from the endosperm using a sharp scalpel blade, immediately frozen in liquid nitrogen, and then stored at ~80 °C. For each extract, 30 embryos were ground in liquid N₂, and total RNA was extracted by a hot phenol procedure according to Verwoerd et al. (1989). RNA concentration was determined spectrophotometrically at 260 nm.

EST database search

In order to analyse the cell cycle transcriptional activity, the expression of the related genes as markers of cell cycle phases was studied (Table 1). The TIGR barley expressed sequence tag (EST) databank (http://www.tigr.org/tigrscripts/tgi/T_index.cgi?species=barley) was screened using specific protein motifs for each class to identify barley genes. To complete and confirm the databank annotation, specific protein motifs were identified using the translation software from infobiogene (http://www.infobiogene.fr/services/analysseq) (Table 1). A class could be attributed for each gene with specific motifs, i.e. for CDKDA (CDKA1), CDKB (CDKB1), CDKD (CDKD1), CYCA (CYCA3), CYCB (CYCB1), and CYCD (CYCD4) (Vandepoele et al., 2002). Specific primers were then designed using the software Primer3 (http://frodo.wi.mit.edu/cgi-bin/primer3/primer3_www.cgi) for each EST (Table 2) to perform the reverse transcription-PCR (RT-PCR) experiments. As an internal standard, a fragment of the barley actin gene (GI24496451) was used. Each expression profile presented was repeated at least three times ± SD.

RT-PCR

Total RNA was treated with DNase I (Sigma, St Louis, MO, USA) and then was reverse transcribed with Revertraid H minus M-MuLV RT (Fermentas). After enzyme inactivation, the first-strand cDNAs obtained were checked by agarose gel electrophoresis. All oligonucleotides were obtained from Eurogentec (Seraing, Belgium). Amplifications were performed using a mastercycler (Eppendorf, Hamburg, Germany) with 1 U of Tag polymerase (New England Biolabs, Beverly, MA, USA), 0.4 mM dNTP, and 0.8 μM of each primer in a 25 μl reaction volume. Semi-quantitative PCRs were initiated at 94 °C for 2 min followed by 25 cycles for CDK genes.

Table 1. Expression pattern of cell cycle genes and their specific protein motifs

<table>
<thead>
<tr>
<th>Gene</th>
<th>Cell cycle expression</th>
<th>Specific motif</th>
<th>EST number</th>
</tr>
</thead>
<tbody>
<tr>
<td>CDKA</td>
<td>Constitutive</td>
<td>EGTY PSTAIRE</td>
<td>TC142283</td>
</tr>
<tr>
<td>CDKB</td>
<td>S/G₁</td>
<td>EGTY PTAIRE</td>
<td>TC141160</td>
</tr>
<tr>
<td>CDKD</td>
<td>G₂/S</td>
<td>NFTALRE</td>
<td>TC133005</td>
</tr>
<tr>
<td>CYCA</td>
<td>G₂/M</td>
<td>LVEVxEY</td>
<td>TC151522</td>
</tr>
<tr>
<td>CYCB</td>
<td>M</td>
<td>HxQxF</td>
<td>TC139865</td>
</tr>
<tr>
<td>CYCD</td>
<td>G₂/S</td>
<td>LxCxE</td>
<td>TC136970</td>
</tr>
</tbody>
</table>
and actin or 30 cycles for cyclin genes, at 94 °C for 30 s, 55 °C for 30 s, and terminated at 72 °C for 5 min. Amplification products were detected on an agarose gel with ethidium bromide and quantified with Quantity One software (Bio-Rad, Hercules, CA, USA). The actin level in each lane was used as an internal standard. Figures 3, 4, and 5 show representative profiles of gene expression obtained in three experiments which were carried out independently.

**ABA quantification**

In parallel with the germination assays, 30 embryos were dissected from the rest of the seed structure and immediately frozen in liquid N₂. The embryos were then lyophilized, powdered, weighed, and stored at −30 °C until assayed for ABA content with a radioimmunoassay as described by Steinbach et al. (1995). The results presented are the means of three biological replicates assayed in duplicate ±SD.

**Table 2. Oligonucleotide sequences used for RT-PCR experiments**

<table>
<thead>
<tr>
<th>Gene</th>
<th>Primer sequence (5’–3’)</th>
<th>Fragment (bp)</th>
</tr>
</thead>
<tbody>
<tr>
<td>CDKA</td>
<td>CTCCTCCCAGCGGCTAC</td>
<td>453</td>
</tr>
<tr>
<td></td>
<td>TTCAGCCCCATGCAACCC</td>
<td></td>
</tr>
<tr>
<td>CDKB</td>
<td>CCCACGCCGCCTCA</td>
<td>473</td>
</tr>
<tr>
<td></td>
<td>CAAATATGCCACCCAGACC</td>
<td></td>
</tr>
<tr>
<td>CDKD</td>
<td>ACTGCATTGAAAGGAATCAAG</td>
<td>434</td>
</tr>
<tr>
<td></td>
<td>ATACACACCAGCGCCAAAAC</td>
<td></td>
</tr>
<tr>
<td>CYCA</td>
<td>GACTGGCTGTCGGAGGTC</td>
<td>440</td>
</tr>
<tr>
<td></td>
<td>TAGGAAAGCCTGCAAACCTCA</td>
<td></td>
</tr>
<tr>
<td>CYCB</td>
<td>GAGGTCCACCGAAGATTGAA</td>
<td>510</td>
</tr>
<tr>
<td></td>
<td>TCCAGTGGAAGGCACTGTTGT</td>
<td></td>
</tr>
<tr>
<td>CYCD</td>
<td>CTCCTGCGCCGAGGACA</td>
<td>418</td>
</tr>
<tr>
<td></td>
<td>CGGAAGTCTCCTCCATCTTG</td>
<td></td>
</tr>
<tr>
<td>Actin</td>
<td>TCACCCGAGAGGGTAACCTCCT</td>
<td>294</td>
</tr>
<tr>
<td></td>
<td>TCCGTATATCCACGCTACACT</td>
<td></td>
</tr>
</tbody>
</table>

**Results**

**Cell cycle activity and ABA content in embryos during seed germination**

All the seed population germinated within 48 h at 30 °C, the time to obtain 50% germination being close to 20 h (Fig. 1A, curve 1). Embryo ABA content was ~430 ng g⁻¹ DW in dry seeds and declined sharply during the first hours of imbibition on water, reaching 135.4 and 94.6 ng g⁻¹ DW after 14 h and 24 h, respectively (Fig. 1A, curve 2).

In dry seeds, nuclei of the embryo radicle tips gave 2C or 4C signals, but 82% of them gave 2C signals, indicating that the majority of cells were arrested at the G₁ phase of the cell cycle (Table 3). Interestingly, the percentages of 4C nuclei were different in the plumule (22%) and in the radicle (8%), suggesting that cell cycle activity is stopped at different phases depending on the organ. During imbibition at 30 °C, the cell cycle was active as soon as 5–8 h (Fig. 1B). The 4C nuclei population increased up to ~60% after 14 h, i.e. after radicle protrusion, and then remained at around this value until 48 h (Fig. 1B, curve 1).

In dry seed, the 4C/2C ratio was only 0.09 in the radicle tips. It increased up to 0.56 after 5 h of imbibition, remained at this value after 8 h, and then increased strongly when radicle protrusion occurred at 14 h, reaching 2.03. The ratio was ~1.3–1.5 after 24 h and 48 h (Fig. 1B, curve 2).

In 24 h germinated seeds, 41, 16, and 43% of the embryo nuclei were present in G₁, S, and G₂ phases of the cell cycle, respectively (Fig. 2A). Incubation of seeds in the presence of 50 mM hydroxyurea, an inhibitor of the S phase (Planche, 2000), did not suppress radicle protrusion (data not shown), but resulted in a blocking of the cell cycle (Fig. 2B). A second peak of nuclei (49%) representing cells re-entering the cell cycle but blocked in the S phase is then observed.

**Cell cycle activity and ABA content of the embryo as related to moisture content**

In order to investigate the effect of moisture content of the embryo on cell cycle activity, the percentages of 2C and 4C nuclei of the radicle tips were determined by flow cytometry after 48 h of incubation of the grains at 30 °C at a water content ranging from 0.11 (dry seeds) to 0.51 g H₂O g⁻¹ FW. ABA contents of the embryos were also measured and the 4C/2C ratios were calculated.

Partial hydration of the grains resulted in a decrease in ABA content of the embryo when the water content was >0.17 g H₂O g⁻¹ FW (Table 4). After 48 h of incubation at 30 °C at low water content (0.11–0.17 g H₂O g⁻¹ FW), ABA content remained high. It decreased with increasing moisture content, reaching 376.7 ng g⁻¹ DW at a water content of 0.28 g H₂O g⁻¹ FW, and then remained close to 200–250 ng g⁻¹ DW at 0.41–0.51 g H₂O g⁻¹ FW (Table 4). Such a value was reached after only 5 h of imbibition on water at 30 °C (Fig. 1A), i.e. when the embryo moisture content was 0.65 g H₂O g⁻¹ FW (data not shown).

**Table 3. Percentages of 4C nuclei and 4C/2C ratio of cells of whole embryos, and of plumules and radicles of embryos isolated from dry seeds**

<table>
<thead>
<tr>
<th>Organ</th>
<th>Nuclei (%) in 4C</th>
<th>4C/2C</th>
</tr>
</thead>
<tbody>
<tr>
<td>Whole embryo</td>
<td>18</td>
<td>0.22</td>
</tr>
<tr>
<td>Plumule</td>
<td>22</td>
<td>0.28</td>
</tr>
<tr>
<td>Radicle</td>
<td>8</td>
<td>0.09</td>
</tr>
</tbody>
</table>

The frequency of 2C and 4C nuclei was calculated as [2C nuclei/(2C nuclei+4C nuclei)]×100 and [4C nuclei/(2C nuclei+4C nuclei)]×100, respectively. The average variation between two measurements of a population of nuclei is ~3%. The populations of 2C and 4C nuclei were measured on 10 000 nuclei.
the 4C/2C ratio, which reached 0.51 at 0.51 g H2Og (Table 4). The higher the moisture content, the higher was Water content (g H2O g⁻¹ FW) and then decreased when the radicle protruded, i.e. at 0.51 g H2Og, while those of CYCA3 and CYCB1 were not detected. CDKA1 expression did not change significantly during seed germination at 30 °C. In contrast, CDKB1 and CDKD1 transcripts progressively accumulated during seed imbibition, and expression of CYCA3 and CYCB1 was observed after 6 h and 12 h of imbibition, respectively. There was a very low expression of CYCD4 throughout germination at 30 °C.

Expression of CDK and CYC during partial hydration of the grain
Partial hydration of the grain at 30 °C at 0.51 g H2O g⁻¹ FW resulted in a lower expression of CDKA1 and CDKB1 and in a higher expression of CDKD1 (Fig. 3B) than the levels detected in embryos isolated from grains incubated on water (Fig. 3A). In contrast, CYCB1 expression was completely suppressed, while that of CYCD4 was induced (Fig. 3B). Expression of CYCA3 was delayed and reduced compared with that in embryos placed on water.

Figure 4 shows the effects of moisture content of the embryo after 48 h of imbibition of the seeds at 30 °C on the expression of CDKA1, CDKB1, and CDKD1, and CYCA3, CYCB1, and CYCD4. CDKA1 was expressed at any moisture content studied, while expression of CDKD1 and CYCD4 increased with increasing moisture content, and then decreased when the radicle protruded, i.e. at 0.65 g H2O g⁻¹ FW. CDKB1 and CYCB1 expression was very low before the radicle protruded from the seed coat, and CYCA3 was highly expressed at 0.51 g H2O g⁻¹ FW when radicle protrusion had not yet occurred.

Effects of ABA on germination, cell cycle activity, and CDK and CYC expression
Figure 5A shows the time-course of germination of non-dormant seeds placed at 30 °C in the presence of 1 mM ABA. Thirty per cent of the seed population germinated within 24 h compared with ~80% for the control seeds placed on water (cf. Fig. 1A), and only ~55% of the seeds germinated within 3 d. This inhibitory effect of ABA was associated with a delay both in germination and in the induction of the cell cycle activity, the 4C/2C ratio being close to 1.08 after 24 h of incubation (data not shown), i.e. when germination occurred.

Changes in expression of CDK and CYC in embryos during the first 24 h of incubation in the presence of 1 mM ABA are illustrated in Fig. 5B. ABA had no significant effect on the expression of CDKA1 and CYCD4 compared with that in embryos of seeds placed

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Changes in expression of CDK and CYC in embryos during the first 24 h of incubation in the presence of 1 mM ABA are illustrated in Fig. 5B. ABA had no significant effect on the expression of CDKA1 and CYCD4 compared with that in embryos of seeds placed
on water (cf. Fig. 3A). It delayed the expression of CDKB1 and CYCB1 as related to the delay in radicle protrusion. In contrast, ABA seemed to improve the expression of CDKD1 slightly as early as 12 h of imbibition and reduced that of CYCA3 from 18 h of incubation in the presence of ABA.

**Discussion and conclusion**

As previously shown by Bino et al. (1993) and Lanteri et al. (1994) in other species, the majority of the nuclei (92%) of the radicle tips of dry mature barley grains are arrested in the cell cycle with a 2C DNA level, and a small number with 4C DNA levels (Table 3). Arrest of nuclei with 4C DNA levels has also been observed in Castanea sativa Miller and Raphanus sativus L. seeds (Bino et al., 1993). In other species, only 2C signals are found in the radicle tips (Cichorium endiva L., Lactuca sativa L., Capsicum annuum L., Pinus nigra), while 4C and also 8C signals are observed in radicle cells of Phaseolus vulgaris L. and Spinacia oleracea L. seeds (Bino et al., 1993). In radicle tips excised from dry embryos of maize, a monocot like barley, 82% of the nuclei had a G1 (2C) DNA content and 17% had a G2 (4C) content (Sanchez et al., 2005).

Flow cytometric analysis of barley radicle tip nuclei shows that cell cycle activity is initiated before radicle elongation (Fig. 1B), the 4C/2C ratio increasing up to 0.56–0.60 after 5–8 h of imbibition (Fig. 1B). Activation of the cell cycle as early as 6 h of imbibition was also observed in embryo radicle tips of maize, the percentage of G2 nuclei increasing to 35% at 15 h (Sanchez et al., 2005). During growth of the radicle, i.e. after 24 h, this ratio was 1.3–1.5 (Fig. 1B), a value similar to that observed in sugarbeet (Sliwinska, 2000). The results presented here also demonstrate that major changes in transcript levels of CDK and CYC genes occurred in the embryo during seed imbibition and the germination *stricto sensu* phase (Fig. 3A). Expression of CDK genes throughout the germination process indicated that the activity of the cell cycle is theoretically possible at the very beginning of imbibition. However, cell cycle progression is regulated by the association of both CDK and CYC units in a protein complex (Dewitte and Murray, 2003), and CYCA3 and CYCB1 transcripts were not present in dry seeds and the level of CYCD4 transcripts remained very low (Fig. 3A). CYCA3 and CYCB1 are expressed as early as 6 h and 12 h of imbibition, respectively, and are fully expressed after radicle elongation (i.e. after 16 h). The pattern of CYCA3 and CYCB1 expression described in Fig. 3A agrees with that observed in Arabidopsis thaliana (Barroços et al., 2005). On the other hand, as for cabbage seeds (Gornik et al., 1997), radicle protrusion is observed in the presence of hydroxyurea, an inhibitor of the S phase (Fig. 2B), suggesting that the cell cycle may not be totally required for the early phases of germination.

Partial hydration of seeds at a moisture content <65% FW did not allow germination of non-dormant barley seeds placed at 30 °C (data not shown); however, it is associated with the reactivation of the cell cycle evaluated by measurement of the percentage of nuclei with 4C DNA levels (i.e. in the G2 phase of the cell cycle) (Table 4); the higher the moisture content, the higher was the 4C/2C ratio (Table 4). It is also correlated with a decrease in ABA content (Table 4). Induction of the cell cycle was also shown during priming treatment of tomato, pepper, sugarbeet, and cauliflower (Bino et al., 1992; Lanteri et al., 1994; Saracco et al., 1995; Özböngöl et al., 1999; De Castro et al., 2000; Sliwinska, 2000).

**Fig. 3.** Expression of the main genes involved in the cell cycle activity. (A) Changes in gene expression during germination at 30 °C on water. Radicle protrusion occurred after 12 h. (B) Changes in gene expression during imbibition of seeds at an embryo water content of 0.51 g H2O g−1 FW.
Plant development is highly influenced by environmental factors. Cell division rates and patterns are highly affected by different factors, such as temperature and availability of water (Bino et al., 1992; De Castro et al., 2000; Boniotti and Griffith, 2002). It is thus necessary for plants to adopt different developmental pathways and integrate cell cycle progression, morphogenesis, growth, and development in response to environmental factors. The results presented here show that controlled hydration of the seeds is associated with changes in expression of CDK and CYC genes, the levels of transcripts depending on water content (Figs 3B, 4). As during germination (Fig. 3A), CDKA1 and CDKB1 transcripts were present in partially imbibed seeds but at lower levels than those observed in embryos of grains placed on water (Fig. 4). CDKD1 expression increased with water content (Fig. 4) and is correlated with the proportion of nuclei shifting from the G1 phase to the S phase (Table 4). Its expression
then decreased in germinating seedlings. Low expression of CDKB1 (a marker of the G2/M transition) in non-germinating embryos is in agreement with the fact that its expression is cell cycle phase dependent (Mironov et al., 1999; Meijer and Murray, 2000; Menges et al., 2005) and suggests that it might play an important role in the regulation of cell division during phase III of the germination process. Lack of expression of CYCB1 (a marker of the M phase) indicated that no mitosis occurred, radicle protrusion allowing the achievement of cell division. In contrast, levels of CYCD4 transcripts (a marker of G1/S transition) were higher in embryos at low water contents, in agreement with the increasing proportion of 4C nuclei. In addition, an increase in CYCD4 transcripts at such a low moisture content suggests that CYCD4 expression did not depend on the complete progression of the cell cycle.

The phytohormone ABA has been implicated in both the imposition and the maintenance of dormancy (Bewley, 1997). A role for ABA in the maintenance of dormancy of the barley grain has also been suggested (Wang et al., 1998a; Benech-Arnold et al., 2006). As in dormant seeds placed in water (Benech-Arnold et al., 2006), ABA embryonic content remained at high levels in partially hydrated non-dormant seeds (Table 4) when it declined during the first hour of incubation on water (Fig. 1A). The remaining high ABA content might result from a lack of ABA leaching into the medium (Wang et al., 1998a) or reduction of ABA through either ABA conjugation or inactivation, in particular through the action of 8’-hydroxylase (Kushiro et al., 2004). Although the embryo ABA content was similar in partially imbibed grains (Table 4) and in dormant grains (Benech-Arnold et al., 2006), ABA was probably not involved in the inability to germinate at a moisture content <0.65 g H2O g−1 FW, since exogenous ABA at 1 mM only slightly delayed germination (Fig. 5A). It also had no strong effect on CDK and CYC expression during the first 24 h (Fig. 5B). Inhibition of germination by ABA was also associated with a delay in cell cycle activation in maize (Sánchez et al., 2005), while it is concomitant with a strong induction of nuclear replication in wild-type and sit8 tomato (Liu et al., 1994). Although ABA did not strongly affect germination of barley non-dormant seeds, it inhibited further radicle growth (data not shown). This inhibitory action probably results from the induction of expression of a cyclin-dependent protein kinase inhibitor (ICK1 actually renamed KRP1 for Kip Related Protein 1) as suggested by Wang et al. (1997, 1998b). Genes of the KRP family might be good candidates to determine whether ABA’s effect on germination of dormant seeds is related to cell cycle progression.

Overall, the results reported here shed new light on the role of water content in the regulation of CDK and CYC expression. They demonstrate that the cell cycle was initiated early before radicle elongation, as previously shown in Arabidopsis (Barróco et al., 2005; Inzé and De Veylder, 2006). It has of course to be considered that transcription may not correlate with translation. The authors’ present work is focused on elucidating whether cell cycle progression and its regulation through ABA might play a role in barley seed dormancy.
References


